Patent Application
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Examiner : C. Chereskin

Art Unit : 1804

Applicants: Michael J. Adang and John D. Kemp

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For : Insect Resistant Plants

Commissioner of Patents and Trademarks
Washington, D.C. 20231

SECOND DECLARATION OF DR. GUY A. CARDINEAU

Sir:

DR. GUY A. CARDINEAU hereby declares:

THAT, my qualifications have been made of record in my first declaration dated April 1992;

THAT, I have reviewed Adang and Kemp application Serial No. 06/535,354 filed September 26, 1983, and the Office Action dated August 26, 1993; and being thus duly qualified, do further declare as follows:

In September of 1983, the state of the art with regard to the cloning and expression of *Bacillus thuringiensis* (Bt) δ-endotoxin insecticidal crystal protein genes in heterologous systems was limited (Schnepf and Whiteley,1981,PNAS78:2893-2897 // Held, et al.,1982, PNAS79:6065-6069 // Klier, et al.,1982). Known crystal proteins were of the approximate size of 130-135kD and were known to undergo processing, after ingestion by a susceptible insect, to a toxic product of 65-68kD (Held, et al.).

The specification of the Adang application describes the cloning of a 6.6kb HindIII fragment derived from a 50 megadalton plasmid of Bt kurstaki HD-73. Both orientations of this insert in the HindIII site of the cloning vector pBR322 were identified and labeled 123/58-3 and 123/58-10. Extracts of *E. coli* strains carrying these clones were used in a bioassay against *Manduca sexta* larvae and were shown to be as toxic as the toxin protein purified from Bt. Sequence analysis of the insert revealed a startling discovery. An open reading frame, corresponding to the Bt ICP coding sequence, contained only 2831 nucleotides before it ran out of the insert and into the vector sequence. Despite the anticipation that a full length δ-endotoxin gene had been cloned, which would code for the 135kD protein, instead a shortened gene had been cloned, the sequence of which can be found in Figure 1 of the application. This insert had no translation termination codon but instead encountered termination codons in frame within 8-11bp on either side of the HindIII site of the vector. Such a gene would code for less than 950 amino acids which would produce a protein of about 105kD. Immunological analysis of cell extracts of these clones via Western blot using antibody against the Bt toxin indicated that neither the 130kD nor a 105kD peptide were produced but, instead, an approximately 67kD peptide, presumably corresponding to the activated toxin domain because of the demonstrated insecticidal activity. This was a <u>significant discovery</u> in that it was the first evidence that a less than full length toxin gene (subsequently referred to by others as a "truncated" toxin gene) was insecticidally active. In fact, it was not until September of

1984. a year after the priority date of the Adang application, that a report of a similar discovery that truncation of Bt toxin genes was possible was made by Helen Whiteley at the Ninth International Spore Conference in Asilomar, CA.

The focus of the Adang application is to express Bt toxin genes in plant cells so as to produce transgenic insect resistant plants. Review of the specification (and supporting notebooks) indicates a variety of different approaches were proposed and evaluated to mobilize the toxin gene into plant cells. These approaches are fully described in the specification. The examples provide alternatives to developing DNA constructions which permit the addition of different plant expressible promoter sequences and sequences which direct the addition of a poly-A sequence at the 3' end of the gene so as to allow expression of the toxin gene in plant cells. Most notably, Example 2 describes the construction of a plant expression cassette based on the use of what is now known as the mannopine synthase promoter from the T-DNA of an Agrobacterium Ti plasmid. The cloning and modification of the mannopine synthase gene to generate a promoter vehicle was performed as described. The modification of the sequence immediately preceding the ATG translational initiation codon of the insecticidal crystal protein gene was performed via oligonucleotide site directed mutagenesis to create a BamHI site to permit insertion of the toxin gene sequence in the mannopine promoter vector. In those cases where adapters or linkers were used to generate new restriction sites, methods well understood by those

skilled in the art at the time of the subject application, all proposed alternatives were not necessarily brought to completion but that approach which succeeded first was carried to the next step. For example, a HindIII site might be converted to a BamHI site by the use of an adaptor with a HindIII "sticky" end on one side and that for BamHI on the other. Alternatively, the HindIII end could be filled in using T4 polymerase and dNTPs and then BamHI linkers added. In those instances where similar alternatives were available, both approaches were attempted concurrently, with that achieving the successful product first taken to the next step. Several different gene constructs were, in fact, developed. These relied on the original identified clone containing the less than full length, or truncated gene, of 2831bp as the starting point to generate full length and several different truncated clones all the way down to the limit truncated product. All of these were identified as producing immunologically reactive proteins that were toxic to insects. Clones which were over shortened were non-insecticidal and were not pursued. A range of insecticidal toxin sequences, from limit truncation to full length, were successfully used, in accordance with the teachings of the specification, to generate insect resistant transgenic plants. The most important consideration was not sequence length but that there be at least sufficient sequence to code for the toxin domain so as to permit production of the toxin in the plant cells.

The mannopine promoter expression vector carrying an insecticidally active Bt toxin gene was mobilized via tri-parental mating into

several Agrobacterium strains. The first vectors, as described in the examples, required co-integration of the promoter/gene construct, via homologous recombination, into the resident T-DNA of the host Agrobacterium strain, a procedure well known in the art at the time. In order to facilitate the regeneration of normal plants, those sequences in the T-DNA of the resident Ti plasmid responsible for tumorigenicity were removed via substitution mutagenesis to produce a "rooty-shooty" mutant of pTi15955 which was designated RS014. Co-integration of the promoter/toxin gene cassette resulted in strains which were subsequently used for tobacco stem inoculations. Calli derived from these transformation experiments were immunologically screened for the presence of the toxin peptide. These tissues were also tested for insecticidal activity. Those tissues that were deemed positive were moved to regeneration medium eventually resulting in transgenic plants expressing the toxin gene. In all cases, both non-transformed tissues and those transformed with only RS014 were used as negative controls.

Subsequent to the use of the co-integrate vectors, an alternative approach involved the use of binary, mini or micro Ti vectors. A description of these types of vectors can be found in the September 1983 specification under the heading "Agrobacterium-manipulations of the TIP plasmids." These vectors, due to their reduced size, were much easier to handle. In my earlier declaration of April 1992, I provided documentation of tobacco plants transformed with a plant expressible Bt toxin gene in Exhibits 2 and 3. These

plants were transformed via Agrobacterium strains carrying a binary vector instead of the co-integrate vector system described above. However, an identical mannopine promoter/Bt toxin gene expression cassette, as described in the specification of the September 1983 application, was used. In both cases, plants were generated expressing an identical Bt toxin gene.

Based on the foregoing, it is clear that the teachings of the September 1983 specification, if followed by the skilled artisan, enable the production of transgenic insecticidal plants.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing therefrom.

Dated: Oct. 22, 1993 Signed: _____